71. ~~The method of claim 70 wherein the amine groups are protected with t-boc or f-moc.~~

72. The kit of claim 53 further comprising one or more protective groups that react with the amine groups in the peptides.

73. The kit of claim 72 wherein the protective groups are t-boc or f-moc.--

---

REMARKS

With this amendment, claims 1-73 are pending.

Amendments to the Specification

The amendment at page 7, lines 18-28 was made to change the word "carboxyamidate" to "amide." This change was made to maintain consistency of language throughout the specification.

The amendment at page 20, lines 21-31 was made to change the format of reference citations for consistency throughout the specification.

All other amendments to the specification were made to correct obvious typographical or grammatical errors. No new matter was added as a result of any amendment to the specification.

Amendments to the claims

Claim 16 was amended to correct a typographical error.

Claim 24 was amended to change the spelling of "phosphoramide." This change was made to correct a typographical error.

Claim 49 was amended to change the phrase "any of claims 48" to "claim 48." This change was made to correct a grammatical error.
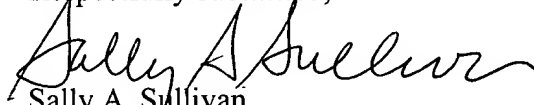
Claims 66 and 67 were amended to add the phrase "one or more protective groups that react with the amine groups in the peptides." This change was made to better clarify the invention as claimed. Support for the addition of this phrase is found, for example, at page 15, lines 10-19 and page 17, lines 15-20.

Claims 68-73 were added to include additional claims of intermediate scope to the use of amine protective groups. Support for the addition of these claims is found, for example, at page 15, lines 10-19 and page 17, lines 15-20.

No new matter was added as a result of the amendments to the claims.

It is believed this amendment does not require the payment of a fee. If this is incorrect, please deduct the appropriate fee from deposit account 07-1969.

Respectfully submitted,

Sally A. Sullivan
Reg. No. 32,064

Greenlee, Winner and Sullivan, P.C.
5370 Manhattan Circle, Suite 201, Boulder, CO 80303
SPhone: (303) 499-8080; FAX: (303) 499-8089
Email: Winner@Greenwin.com
Attorney Docket No. 39-00
lem:10/12/01

19

Page 4, first full paragraph:

Protein phosphorylation is one of the most important regulatory events in cells. The state of activity of numerous enzymes and processes and the association of specific proteins into functional complexes are frequently controlled by reversible protein phosphorylation (Graves, J.D. & Krebs, E.D. (1999), "Protein phosphorylation and signal transduction," *Pharmacol. Ther.* **82**, 111-121; Koch, C. A. et al. (1991), " SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins," *Science* **252**, 668-674; Hunter, T. (1994), "1001 protein kinases redux--towards 2000," *Semin. Cell Biol.* **5**, 367-376). The principle goals of studying protein phosphorylation are the identification, quantitation and determination of the biological function of phosphorylation site(s) in phosphoproteins. Much of the difficulty in such studies lies in the fact that many phosphoproteins exist [at] only at very low abundance. Further, proteins are often phosphorylated at a low stoichiometry and at multiple sites. Therefore, it is usually difficult to obtain sufficient amounts of pure phosphoprotein for such analyses. All current methods for the analysis of the phosphorylation state of proteins focus on one purified phosphoprotein at a time (Verma, R. et al. (1997), "Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase," *Science* **278**, 455-60; Watts, J.D. et al. (1994), "Identification by electrospray ionization mass spectrometry of the sites of tyrosine phosphorylation induced in activated Jurkat T cells on the protein tyrosine kinase ZAP-70," *J. Biol. Chem.* **269**, 29520-29529; Gingras, A.C. et al. (1999), "Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism," *Genes Dev.* **13**, 1422-1437). Because cellular proteins are coordinately phosphorylated to control specific biological processes, the complex mechanisms that control biological systems by protein phosphorylation are difficult to investigate using current technology.

Page 5, second full paragraph

This invention provides a method for selective labeling of phosphate groups in natural and synthetic oligomers and polymers in the presence of chemically related groups such as carboxylic acid groups. The method is specifically applicable to biological oligomers and polymers, including phosphopeptides, phosphoproteins and phospholipids. Selective labeling of phosphate groups in proteins and peptides, for example, facilitates separation, isolation and detection of phosphoproteins and phosphopeptides in complex mixtures of proteins. Selective labeling can be employed to selectively introduce phosphate labels at phosphate groups in an oligomer or polymer, e.g., in a peptide or protein. Detection of the presence of the label[,] is used to detect the presence of the phosphate group in the oligomer or polymer. The method is of

particular use for the detection of phosphoproteins or phosphopeptides. The phosphate label can be a colorimetric label, a radiolabel, an isotope label, a fluorescent or phosphorescent label, an affinity label or a linker group carrying a reactive group (or latent reactive group) that allows selective attachment of the oligomer or polymer (protein or peptide) to a phosphate label, to an affinity label or to a solid phase material.

Paragraph bridging pages 5 and 6

Selective attachment of a phosphate group to its affinity label or selective attachment to a solid support allows selective isolation of an oligomer or polymer (e.g., protein or peptide) that carries at least one phosphate group. The presence of an affinity label allows capture of the selectively labeled oligomer or polymer using a capture reagent that specifically bonds to the affinity label. The presence of a linker that allows selective covalent attachment of the oligomer or polymer to a solid surface allows physical separation of the selectively labeled oligomer or polymer from non-selectively labeled (non-phosphorylated) species in a sample. The method is particularly useful for the selective[ly] labeling of proteins and peptides having a phosphate group (phosphoproteins and phosphopeptides) in the presence of carboxylic acid groups. The method of this invention can be used specifically to separate phosphoproteins and phosphopeptides from mixtures of non-phosphorylated proteins and/or nonphosphorylated peptides and thus to overcome detection problems due to low levels of these species in samples.

Paragraph bridging pages 7 and 8

In a preferred embodiment, selective labeling of phosphate groups is accomplished by initial reaction of the proteins and peptides in one or more samples with a protective group, such as an amine, that reacts in the presence of a condensation catalyst with and protects both carboxylic acid groups and phosphate groups. Amines react with carboxylic acid (or the corresponding esters) to form [carboxyamidate] amide bonds. Amines react with phosphates or phosphate ester groups to form phosphoramide bonds. The labeled phosphoramide bonds in the protected proteins and peptides are then selectively cleaved with a reagent that does not cleave [carboxyamide] amide bonds. This results in the regeneration of free phosphate groups which can be selectively labeled or linked to a solid surface. In a specific embodiment, an amine, such as ethanolamine, can be used for initial protection of all carboxylic acid and all phosphate groups. For example, a carbodiimide catalyzed condensation of an amine with the peptide or protein forms amide and phosphoramide bonds. Ethanolamine can be selectively cleaved from the phosphate groups of the protein or peptide by treatment with mild acid conditions (e.g., trifluoroacetic acid (tfa), 10-30% by volume in water, exemplifying mild acid conditions).

21

Excess protecting agent (e.g., excess amine) can be removed by extensive washing of the peptides on a reverse phase column. In specific embodiments, the free phosphate is reacted with a linker group that carries a reactive functional group including a latent reactive group (such as a sulfhydryl group) that can be used to attach the phosphoprotein or phosphopeptide to a solid support or used for selective labeling of phosphopeptides and phosphoproteins with a phosphate label. For example, carbodiimide-catalyzed condensation reactions can be used to attach cystamine to free phosphate groups. The disulfide bond in the cystamine can be cleaved to generate a reactive sulfhydryl group. (Cystamine is an example of a group that carries a latent reactive group).

Page 9, second full paragraph

The label may be an affinity label and, if so, the kit preferably contains a capture reagent appropriate for use with the affinity label. A kit optionally contains protective groups for amines (e.g., t-boc or f-moc), and solid phase materials. The kit may further contain a set of differentially isotopically labeled protective groups, linkers, affinity labels, or other labels (fluorescent, chromophoric or phosphorescent) to allow quantitative determination of the amounts (or relative amounts) of phosphoproteins and phosphopeptides in different samples. With respect to fluorescent, chromophoric, radiolabels or other labels, different types of labels can be used to label the phosphates in different samples. For example, different fluorescent labels that are separately detectable and can be individually quantitated (e.g., fluorescein amine, rhodamine amine) can be used to label different samples and to detect relative amounts of labeled peptides in difference samples. Kits further optionally contain instructions for carrying out selective labeling, as well as[.] directions for conducting various types of analysis that can be used in combination with the kit to detect, identify, or quantitate phosphopeptides and phosphoproteins.

Page 10, first full paragraph

Reagents which substantially cleave one bond in the substantial absence of cleavage [in] of the other exhibit at least about a 10:1 ratio of cleavage of one bond to another (measured in terms of the rate of reaction or the amount of cleavage product detected) and preferably exhibit at least about a 20:1 ratio, and more preferably, at least about a 100:1 ratio of cleavage of one bond to the other. Of course for applications to the methods herein, the reagents for selective cleavage of bonds are preferably chosen to cleave one bond without any measurable cleavage of the other bond.

22

Page 10, second full paragraph

In this method the phosphopeptide or phosphoproteins can be covalently attached to a solid support material through reaction with a sulfhydryl group of the linker and the solid support can comprise immobilized iodoacetyl groups for reaction with sulfhydryl groups. In this method phosphopeptides or phosphoproteins [an] can be separated from a mixture by attachment to a solid support or by binding of the phosphopeptides to a capture reagent via an affinity label.

Page 15, first full paragraph

This invention provides methods for detecting the presence of phosphopeptides and phosphoproteins in a mixture, identifying phosphopeptides and phosphoproteins that are present in a mixture and determining the relative amounts of phosphopeptides and phosphoproteins in one or more mixtures. The methods are based on the ability to selectively [from] form covalent bonds to phosphate groups of peptides in the presence of carboxylic acid (or ester) groups and amine groups of peptides. The methods are more specifically based on the ability to selectively bond a label or linker to a phosphate group in the presence of carboxylic acids. In particular, the method relies on the ability to cleave phosphoramide bonds under mild acid conditions which do not cleave amide bonds. Thus, selective labeling or linking to a phosphate group in the method proceeds by initial conversion of peptide and protein carboxylic acid groups (of the C-terminus and amino acid side groups) to amides and the conversion of phosphate groups of peptides and proteins to phosphoramidate. Thereafter, the phosphoramidate are selectively cleaved without cleaving the amides and the free phosphate groups are reacted with selected labels or linkers to facilitate detection, identification and quantitation of phosphopeptides in one or more samples.

Page 15, second full paragraph

The peptides which are amine-protected and in which the carboxylic acid groups and any phosphate groups have been converted to amides and phosphoramidates, respectively, are then treated under mild acid conditions to [selective] selectively cleave the phosphoramide bonds leaving the amide bonds substantially intact. Any phosphopeptides in a sample carry free phosphate groups and the phosphopeptides in different samples are differentially isotopically labeled. The phosphopeptides in each sample can then be selected attached to a solid support, via a sulfhydryl linker generated by condensation of the phosphate groups with cystamine. The attached cystamines are then reduced to generate free sulfhydryl groups which can react with iodoacetyl groups on a solid support. The cystamine functions as a latent reactive group which is activated for attachment to the solid support by reduction (e.g., with dithiothreitol, DTT or tris[2-carboxlethylphosphine], TCEP).

23

Page 16, first full paragraph

Phosphopeptides in samples are attached to the solid support and after extensive washing are cleaved off the support (e.g., using trifluoroacetic acid). Preferably, the amine protective groups are cleaved in this reaction as well. These steps provide [purifies,] purified, isotopically labeled phosphopeptides which [an] can be subjected to tandem mass spectrometric analysis. CID mass spectra provide the sequence of any phosphopeptide present in a sample and indicate the presence and location of phosphorylated amino acid residues. Using the peptide sequence information obtained, database searching can be performed to determine the protein source of the phosphopeptides detected. The relative signal intensities of differentially isotopically labeled phosphopeptides in different samples detected in mass scans in the same mass spectrometer allow measurement of the ratio of abundance of the labeled phosphopeptides in different samples.

Paragraph bridging pages 16 and 17

--   Covalent linkage of the amine labeling the phosphate group to a solid support is a preferred implementation of the method of this invention because it allows stringent washing of the immobilized phosphopeptides and their specific release by acid treatment. The use of cysteine to label phosphate groups is preferred because any peptide with residual unblocked carboxylic acid groups, whether it contains phosphate or not, will be converted into sulfhydryl groups and remain attached to the solid support irreversibly. The presence of cysteine residues interferes with the above method, however, cysteine groups can be optionally alkylated by iodoacetamide or any other known alkylating reagent. Before any protein sample is subjected to this method of phosphopeptide isolation, it can be reduced by dithiothreitol, and then alkylated by excess of iodoacetamide under denaturing condition. The solid phase- based embodiment of the method of this invention therefore serves as an efficient way for highly specific phosphopeptide purification and the stable isotope tags introduced by carboxyl group derivatization serves as the basis for relative phosphopeptide quantitation. Any solid material that can be derivatized with functional groups that facilitate attachment of the phosphopeptides (e.g., via sulfhydryl groups) and that is otherwise relatively inert with respect to the peptides, reagents and washing conditions of the method can be used. For example, any solid phase material that is useful in solid phase peptide synthesis can be employed. Glass beads are a preferred solid phase material.

Page 17, first full paragraph

24

With respect to fluorescent, chromophoric, radiolabels or other labels, different types of labels can be used to label the phosphates in difference samples. For example, different fluorescent labels that are separately detectable and can be individually measured (e.g., fluorescein amine, rhodamine amine) can be used to label different samples and to detect relative amounts of labeled peptides in different samples. These labels can also be used to separate the peptides by RP-HPLC or CE (capillary electrophoresis) and to detect the relative amounts of peptides by fluorescence measurement. To implement quantitative measurement of relative amounts of peptides in [difference] <u>different</u> samples, it is preferred to calibrate the system to account for differences in detection of the different labels. For example, is it preferred when using different fluorescent labels to calibrate for differences in quantum yields of different labels.

Paragraph bridging pages 17 and 18

Figure 1 illustrates the selective labeling method of this invention. As illustrated, peptides (1) are first reacted with an amine protective group (2), such as tBoc (t-Butoxydicarbonate). A variety of useful amine protective groups are known in the art and readily available for application in this method. The protective group selected must be compatible with other reagents used in the method. Peptides with protected amine groups (3)are then treated with a reagent that reacts to protect carboxylic acid and phosphate groups (4), such as an amine. An amine group will react with a carboxylic acid group to form an amide (-CO-NH-) bond or with a phosphate group to form a [phosphoramidate] <u>phosphoramide</u> ($-PO_2-NH-$) bond. The fully protected peptide (5) is treated to selectively cleave the phosphoramide bond to remove protection from the phosphate group. Mild acid conditions (6) are used to selectively regenerate the phosphate group. For example, the protected peptide can be treated with trifluoroacetic acid (tfa) (about 30% or less by volume in water for about 1 hour, preferably about 20% in water for about 1 hour, and more preferably 10% for 30 min. at room temperature). Mild acidic conditions include the use of a strong acid, such as tfa, in diluted form. Other mild acidic conditions that will achieve the desired chemistry can be determined by routine experimentation <u>in</u> which treatment is varied to maximize phosphoramide cleavage and minimize amide cleavage.

Page 18, first full paragraph

Protected peptides in which the phosphate protective groups have been removed (7) are treated to selectively label the free phosphate group. For example, a linker containing a sulfhydryl (8) or other reactive group can be selectively bonded to the phosphate groups. The linker group contains functionality for bonding to the phosphate group (e.g., an amino group) and

functionality for linking to a label or to a solid surface. Alternatively, at this point in the method, a label (an affinity label or any phosphate label such as a fluorescent label or radiolabel) carrying functionality for bonding to the phosphate group can be directly attached to the phosphate. As illustrated in Fig. 1, the functionality on the linker group may be a latent functional group which must be activated prior to reaction to add a label or to [covalent] covalently attach to a solid surface. In the illustrated case, cystamine carries a latent sulfhydryl functional group (a -S-S- bond) which is activated by addition of a reducing agent (9, such as TCEP or DTT) that reduces disulfide bonds.

Paragraph bridging pages 18 and 19

Figure 1 illustrates a step of treating the bound phosphopeptides with hydroxylamine (14). More specifically, beads carrying bound peptide (13) are washed and incubated in 1M hydroxylamine (14, pH 10.0) for about 2 hrs. to regenerate tyrosine, since tyrosine residues can form adducts with carbodiimide. This step is [optionally,] optional, but preferred, when carbodiimide reagents are employed in the method, to regenerate tyrosines in the bound peptide which may have formed adducts with the carbodiimide reagent. It has been found that treatment with 5% hydroxylamine for about 30 min. is sufficient to regenerate tyrosine.

Paragraph bridging pages 20 and 21

The following references relate to the application of mass spectrometric techniques to protein identification, particularly with respect to proteome analysis: [Ideker T, Thorsson V, Ranish JA, Christmas R, Buhler J, Eng JK, Bumgarner R, Goodlett DR, Aebersold R, Hood L "Integrated genomic and proteomic analyses of a systematically perturbed metabolic network." Science. 2001 May 4;292(5518):929-34; Gygi SP, Aebersold R. "Mass spectrometry and proteomics." Curr Opin Chem Biol. 2000 Oct;4(5):489-94.; Gygi SP, Rist B, Aebersold R "Measuring gene expression by quantitative proteome analysis" Curr Opin Biotechnol." 2000 Aug;11(4):396-401; Goodlett DR, Bruce JE, Anderson GA, Rist B, Pasa-Tolic L, Fiehn O, Smith RD, Aebersold R."Protein identification with a single accurate mass of a cysteine-containing peptide and constrained database searching."Anal Chem. 2000 Mar 15;72(6):1112-8.; and Goodlett DR, Aebersold R, Watts JD."Quantitative in vitro kinase reaction as a guide for phosphoprotein analysis by mass spectrometry."Rapid Commun Mass Spectrom. 2000;14(5):344-8; Zhou, H. et al (Apr 2001) Nature Biotechnol. 19:375-378.] Ideker T., et al. (May 4, 2001), "Integrated genomic and proteomic analyses of a systematically perturbed metabolic network," Science 292(5518):929-34; Gygi S.P. and Aebersold R. (Oct 2000) "Mass spectrometry and proteomics," Curr Opin Chem Biol. 4(5):489-94.; Gygi, S.P. et al. (Aug 2000)

"Measuring gene expression by quantitative proteome analysis," *Curr Opin Biotechnol.*
11(4):396-401; Goodlett, D.R. et al. (May 15, 2000) "Protein identification with a single accurate
mass of a cysteine-containing peptide and constrained database searching,"*Anal Chem.*
72(6):1112-8.; and Goodlett, D.R. et al., (2000) "Quantitative in vitro kinase reaction as a guide
for phosphoprotein analysis by mass spectrometry," *Rapid Commun Mass Spectrom.* 14(5):344-
8; Zhou, H. et al (Apr 2001) *Nature Biotechnol.* 19:375-378. These reference are incorporated
by reference herein to the extent that they are not inconsistent with the disclosure of this
application.

Page 21, second full paragraph

        Amines are preferred reagents for selective labeling of carboxylic acids in the presence of
phosphate groups. Any amine reagent will generally provide the function of this protective
group. [Alkanolamine,] Alkanolamines, such as ethanolamine are preferred amine reagents.
One of ordinary skill in the art will appreciate that other reagents can be found which provide a
similar selective labeling function. One or ordinary skill in the art can identify and [selection]
select other reagents for selectively labeling without resort to undue experimentation. The
protective group employed must, however, also be suitable for use in the reactions of this
invention. Any art-known methods and reagents suitable for selective protection of carboxylic
acid groups as described herein are intended to be encompassed by this invention. With respect
to amines that are used to selectively protect carboxylic acid groups, the reaction of amines with
carboxylic acids and phosphate groups is preferably done in the presence of a coupling agent.
Coupling agents that can be used in this reaction include, among others,
dicyclohexylcarbodiimide, or 2,3,5,6-tetrafluorophenyl trifluoroacetate. In addition, a coupling
catalyst such as 4-dimethylaminopyridine can be employed.

Page 22, second full paragraph

        The method of this invention as specifically exemplified employs steps of washing
peptides on [Reverse Phase] reverse phase columns to remove undesired materials from the
peptide sample. Those of ordinary skill in the art will appreciate that methods for removing such
materials other than those specifically described herein are known in the art and can be readily
applied to the method herein to achieve the desired result. All such art-known methods for
washing or removal of undesired materials are intended to be encompassed by this invention.

Paragraph bridging pages 22 and 23

The sequence of a phosphopeptide and the identification of the site(s) of phosphorylation can be determined by a combination of tandem mass spectrometry and computer-assisted database search programs, such as SEQUEST (Trademark, University of Washington, Seattle WA) (McCormack, A.L. et al. (1996) "Direct Analysis and Identification of Proteins in Mixtures by LC/MS/MS and Database Searching at the Low-Femtomole Level", Anal. Chem. 69, 767-776; Eng, J.K. et al. (1994)"An Approach to Correlate Tandem Mass Spectral Data of Peptides with Amino Acid Sequences in a Protein Database" J. Amer. Soc. Mass. Spectrom., 5, 976-989; U. S. Patent 5,538,897 (Jul. 23, 1996) Yates, III et al.) In the first stage of a tandem mass spectrometer, any given phosphopeptide is selected and subjected to collision induced dissociation (CID). The spectra of a resulting fragment ion is recorded in the second stage of the mass spectrometry, as a so-called CID spectrum. This process is repeated with other (ideally all) peptides present in the sample. Because the CID process usually causes fragmentation at peptide bonds and different amino acids for the most part yield peaks of different masses, a CDI spectrum alone often provides enough information to determine a peptide sequence. Peptide sequencing and protein identification is facilitated by using a sequence searching computer program, such as SEQUEST™, which takes all known genomic [sequence,] sequences, computes all possible theoretical CID spectra and compares them to experimental CID spectra for matches and sequence identification. The mass modification to the C-terminus, glutamic acid, aspartic acids and any other acidic side groups are known and this information can be incorporated into the computer analysis. Also mass changes due to phosphorylation are also known and can be incorporated into the computer analysis. Data can be searched for any possible phosphorylations to serine, tyrosine, and threonine residues, thus allowing the identification of sites of phosphorylation.

Page 27, second full paragraph

DL itself preferably is minimally ionized during mass spectrometric analysis and the formation of ions composed of DL clusters is preferably minimal. The selection of DL[,] depends upon the A and CR groups that are employed. In general, DL is selected to displace A from CR in a reasonable time scale, at most within a week of its addition, but more preferably within a few minutes or up to an hour. The affinity of DL for CR should be comparable or stronger than the affinity of the tagged compounds containing A for CR. Furthermore, DL

should be soluble in the solvent used during the elution of tagged compounds containing A from CR. DL preferably is free A or a derivative or structural modification of A. Examples of DL include, d-biotin or d-biotin derivatives, particularly those containing groups that suppress cluster formation or suppress ionization in MS.

Paragraph bridging pages 29 and 30

Example 1:Phosphopeptide Isolation Procedure

Peptide samples were dried, and then subjected to the method shown in Fig. 1A according to the following steps. 1) Peptide mixture was resuspended in 50% (v/v) of 0.1 M phosphate buffer (pH 11)/acetonitrile. 0.1 M of t-Butyl-dicarbonate (tBoc) was added for 4 hours at room temperature. 2) Acetonitrile was removed under reduced pressure. Samples were made to 1 M ethanolamine, 25 mM N-hydroxysuccinimide (NHS) and 0.5 M of N,N'-dimethylaminopropyl ethyl carbodiimide HCl (EDC) and incubated 2 hours at room temperature. 3) 10% trifluoroacetic acid (TFA) was added for 30 minutes at room temperature. Longer treatment under these conditions did not detrimentally [effect] affect the results. Samples can be neutralized at this point, but neutralization was found to have no significant effect on results. Samples were then desalted on and recovered from a C18 column (Waters Associates, Milford, MA WAT 023590) using elution with 80% acetonitrile, 0.1% TFA. 4) Peptides were dried and redissolved in 1 M imidazole (pH 6.0). Imidazole is optional and was employed to inhibit possible carbodiimide adduct formation with sensitive amino acids such as histidine. 0.5 M EDC was added for 3 hours at room temperature. Samples were loaded on a C18 column, washed with water and treated with 1 M cystamine (pH 8.0) for 2 hours at 50 °C on the column. Peptides were washed with water and reduced with 10 mM DTT to generate free sulfhydryl groups. 5) After washing to remove DTT, peptides were eluted with 80% acetonitrile, 0.1% TFA and incubated with 20 mg beads with immobilized iodoacetyl groups for at least 2 hours at pH 8.0 (titrated with 1 M Tris pH 8.0, 50 mM EDTA). Beads with immobilized iodoacetyl groups were prepared by a 2-hour reaction between 3 equivalents of iodoacetic anhydride and 1 equivalent of amino beads (Sigma, G4643) with 3.3 equivalent of diisopropylethylamine in dimethylformide. The formation of a tyrosine adduct with carbodiimide is a possible side reaction. Such an adduct is unstable against nucleophiles such as hydroxylamine. Therefore, after attachment of phosphopeptide to the beads, 1 M hydroxylamine (pH 10) was used to incubate beads for 2hours at room temperature. This restored tyrosine residues. It has been found that treatment with 5% hydroxylamine solution for 30 min. is typically sufficient to restore tyrosine residues. Beads were then washed sequentially with 2 M NaCl, methanol and water to remove

nonspecifically bound molecules. 6) The beads were incubated with 100% TFA for 30 minutes to recover phosphopeptides. Concurrently, tBoc protection was removed. The recovered sample was dried under reduced pressure and resuspended in water for LC-MS/MS analysis.

Page 30, first full paragraph

Two separate samples of equal amounts of phosphoangiotensin peptide were analyzed by the method of this invention. The carboxylic acid groups in the two different samples were blocked (leaving phosphate groups free as described above) by either light ethanolamine (d0-ethanolamine) or heavy ethanolamine (d4-ethanolamine, $HOCD_2CD_2NH_2$). Phosphoangiotensin contains two carboxylic acid groups, so that the mass difference for the $[M+2H]^{2+}$ ion is 4 for the differentially labeled peptides. The results of mass spectrometric analysis of the differentially labeled samples that were subjected to selective labeling and separation of phosphopeptides of this invention [is] are illustrated in Figs. 3A-C. A doublet of peaks $[M+2H]^{2+}$ at m/z≅ 607 and 611, due to light and heavy labeled samples, respectively, is observed as expected. Further the relative ratios of the two peaks is about 1:1 as expected. The CID spectrum of each of these peaks is similar to that of the unprotected peptide, except for the fragment ions that are mass shifted by the modification (label attachment). Modifications to the carboxylic acid groups used to achieve differential labeling do not adversely affect the quality of the CID spectrum that would be used to identify the sequence of unknown peptides

Page 31, line 10

Example [4]3: Isolation of Phosphopeptides from β-casein

Page 32, third full paragraph

The ion chromatogram for the isolated phosphopeptides is illustrated in Fig. 5A where the most prominent ion at m/2 = 630.1 (2+) was chosen for fragmentation. The CID of this ion is shown in Figure 5B. This unambiguously identified the phosphopeptide as THY*GSLPQK of [M.P.] M.B.P.(Aebersold, R. et al. (1991), "Determination of the site of tyrosine phosphorylation a the low picomole level by automated solid-phase sequence analysis," Anal. Biochem. 199:51-60). Phosphopeptide recovery efficiency over the entire six-step procedure was assessed by measuring radioactive counts recovered after each step of the procedure, with a final yield that was consistently about ~20% of the starting material.

Page 33, second full paragraph

Yeast *S. cerevisiae* strain (BWG1-7A) was grown till mid-log phase on YPD media with 2% glucose as a carbon source and harvested by centrifugation. Protein extract was prepared by the glass beads method as described in *Current Protocols in Molecular Biology* (New York, J. Wiley). A mixture of DNAse 1 (20 U/ml) and RNAse (10 μg/ml) was added for 30 minutes on ice. Protein concentration was determined using Biorad protein assay and 500 μg of the protein extract was then denatured in 0.1 M potassium phosphate buffer (pH 8.0) with 6 M urea. Proteins were reduced and alkylated by addition of DTT (10mM, 30 [min)followed] min) followed by 2 hours of incubation with iodoacetamide. Samples were then dialyzed prior to digestion overnight with trypsin at 37 °C. The resulting peptide mixture was desalted by reverse phase C18 column as described above. Samples were treated as in Example 1.

Paragraph bridging pages 33 and 34

Phosphopeptides were isolated by this method and analyzed by LC-MS/MS, with CID spectra being recorded and searched against the yeast sequence database. Fig. 6A shows the total ion intensity recorded with respect to retention time on the column, indicating the complexity of the sample. Fig. 6B shows the *m/z* values observed integrated over the time window indicated in Fig. 6A. The major peptide peaks observed which also displayed loss of 98 Da during CID are labeled with an asterisk (*), confirming that the majority of the peptides detected were indeed phosphorylated. [Further more,] Furthermore, the selectivity of the method was apparent by virtue of the fact that over 80% of the CID spectra that led to identification were derived from phosphopeptides. Additionally, CID spectra derived from the few non-phosphorylated peptides identified generally resulted from lower intensity precursors ions. Thus even with a highly complex starting material, only low level of non-specific peptide background carried through the isolation procedure to the MS, affirming its selectivity. In the example shown, the ion at *m/z*=1032.7 in Fig. 6B was selected for CID, this spectrum being shown in Fig. 6C. In addition to observing a clear fragment ion series, a major signal corresponding to the doubly charged parent ion after undergoing the loss of H_3PO_4 is apparent at *m/z*=983.8.

31

Page 34, first full paragraph

Following database searching, the peptide was identified as being from enolase and having the [indicated sequence] sequence indicated in Table 1. This peptide contained three potential threonine phosphorylation sites, and the parent ion mass indicated that the peptide contained a single phosphate group. The y-7 to y-13 ions confirmed that the phosphate was not on the N-terminal threonine. Two possible pairs of the y-5 and y-6 ions correspond to either one of the other two threonine residues being phosphorylated. Thus the exact site of phosphorylation for this peptide could not be determined. Further, the possibility that a mixture of both possible mono-phosphorylated species of this peptide may have co-eluted from the LC column can not be excluded.

Page 35, first full pargraph

No peptides with multiple serine or threonine phosphorylation sites were identified. In many cases, ions corresponding to loss of H_3PO_4 dominated the fragmentation process, resulting in insufficient fragmentation at peptide bonds for sequencing. This effect would be compounded by multiple phosphoserine[-] or phosphothreonine sites in a single peptide. Peptides of too large or small sizes are generally not suitable for MS sequencing; additionally, such peptides could be lost during the desalting steps in this method. Whether this method could completely determine all the phosphorylation sites of a given protein therefore depends on whether phosphorylation sites are contained in peptides of suitable sizes/hydrophobicities for MS analysis, a limitation common to all MS-based methods. In such cases, alternative proteolytic enzymes can be considered. In the experiment on yeast lysate, tyrosine phosphorylated peptides were not identified, likely due to its significantly lower abundance.

16.   The method [ofclaim1] of claim 1 wherein the label or tag is an affinity label.

24.   The method of claim 23 wherein the carboxylic acid groups and phosphate groups of the peptides are initially protected with an amine group that forms amide bonds with carboxylic acid groups and phosphoramide bonds with phosphates and wherein the phosphate groups are selectively deprotected by selective cleavage of the [phosphoamide] phosphoramide bonds.

49.   The method of [any of claims 48] claim 48 wherein the samples are protein digests containing peptides and the sequence of the phosphopeptide detected is used to identify the protein from which the phosphopeptide is derived.

65.   The kit of claim 53 further comprising one or more enzymes for carrying out a peptide[.] digest.

66.   The kit of claim 53 which comprises:

        one or more enzymes for carrying out a peptide digest;

        one or more protective groups that react with carboxylic acid or carboxylic acid ester groups and with a phosphate group;

        one or more protective groups that react with the amine groups in the peptides;

one or more reagents for selectively cleaving a phosphoramide bond in the presence of amide bonds to generate free phosphates; and

one or more labels which carry a functional group for selectively reacting with free phosphate groups.

67.   The kit of claim 53 which comprises:

one or more enzymes for carrying out a peptide digest;

one or more protective groups that react with the amine groups in the peptides;

one or more protective groups that react with carboxylic acid or carboxylic acid ester groups and with a phosphate group;

one or more reagents for selectively cleaving a phosphoramide bond in the presence of amide bonds to generate free phosphates; and

one or more linkers which carry a functional group for selectively reacting with free phosphate groups and carry a reactive group or a latent reactive group for reacting with a solid support material;

one or more solid support materials to which the reactive group or the latent reactive group can be attached; and

one or more reagents for cleaving a phosphopeptide from a solid support material.

68.   The method of claim 1 further comprising a step of protecting the amine groups in the peptide or protein prior to step a.

69.   The method of claim 68 wherein the amine groups are protected with t-boc or f-moc.

70.   The method of claim 22 further comprising a step of protecting the amine groups in the peptide or protein prior to step a.

71.   The method of claim 70 wherein the amine groups are protected with t-boc or f-moc.

72.   The kit of claim 53 further comprising one or more protective groups that react with the amine groups in the peptides.

73.   The kit of claim 72 wherein the protective groups are t-boc or f-moc.